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Biophysical studies on the base specificity and energetics of the DNA interaction of photoactive dye thionine: Spectroscopic and calorimetric approach

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ABSTRACT

In this study absorbance, fluorescence, circular dichroic spectroscopy, viscosity, thermal melting and calorimetric techniques were employed to understand the binding of the phenothiazinium dye, thionine, with deoxyribonucleic acids of varying base composition. Strong hypochromic and bathochromic effects and quenching of fluorescence were observed that showed strong binding of thionine to the DNAs. The binding parameters evaluated from Scatchard analysis through McGhee–von Hippel analysis showed that the binding was non-cooperative and affinities of the order of 10^5 M^{-1}. The results of ferrocyanide fluorescence quenching studies and viscosity experiments, taken together suggested the intercalation of thionine while thermal melting, differential scanning calorimetry and circular dichroic studies provided evidence for the thermal stabilization and conformational perturbations associated with the binding. The thermodynamic parameters elucidated through sensitive isothermal titration calorimetric studies suggested that the binding was exothermic, characterized by negative enthalpy and large positive entropy changes and that the non-electrostatic contributions play a significant role for thionine association to DNA. The heat capacity changes obtained from the temperature dependence of enthalpy changes gave negative values suggesting substantial hydrophobic contribution in the DNA binding process of thionine. Further, an observation of enthalpy–entropy compensation in the DNA binding also suggested the involvement of multiplicity of non covalent interactions in the binding process. The base specificity of the complexation and energetics of the interaction of thionine to DNA are obtained for the first time from this study.

1. Introduction

Interaction of heterocyclic aromatic molecules with DNA is an interesting topic that has relevance and implications in several biological applications including cancer chemotherapy [1–7]. For example, in anticancer therapy specific interaction of the molecule with the genomic DNA may be effective in preventing further information retrieval from DNA leading to the arrest of cell division. Another non invasive therapeutic action of such molecules may also involve photosensitization involving energy transfer from a photoexcited molecule to molecular oxygen generating a singlet state that may cause direct or indirect damages to the DNA in the cells. Such photophysical behaviour linked to DNA binding could be used for site directed cleavage of the DNA backbone for photodynamic therapy of tumors and other diseases [8,9]. For these and other related reasons, molecular aspect of the interaction of many aromatic molecules that essentially bind by intercalation with DNA has been the subject matter of a large number of investigations in the past 50 years or so. The intercalative binding properties of such molecules can also be harnessed as diagnostic probes for DNA structure in addition to DNA directed therapeutics [1,6,7,10]. Nevertheless, the studies still now have remained inconclusive in terms of a comprehensive understanding the nature, specificity and energetics of the interaction and the development of effective chemotherapeutic agents remained, to a large extent, an elusive goal and still challenging. It appears that more concerted effort is required to understand the molecular aspects and energetics of the interaction of the binding reaction as a function of base sequence and selectivity.

Thionine (3,7-Diamino-5-phenothiazinium) (Fig. 1A), a tricyclic heteroaromatic molecule, has been studied for its intercalative interaction, photoinduced mutagenic actions on binding to DNA, photoinduced inactivation of viruses and as impedance based DNA biosensor [11–14]. Tuite and Kelly [15] suggested that thionine intercalated and also bound to the outside of linear duplex DNA. Their studies also suggested that the guanine–cytosine (GC) specificity of thionine binding was not very marked. On the contrary, Chang et al. [16] suggested a GC specific binding from satellite hole spectroscopy study. It was further suggested [17,18] that thionine exists in two different tautomeric forms and the amino form is intercalated while

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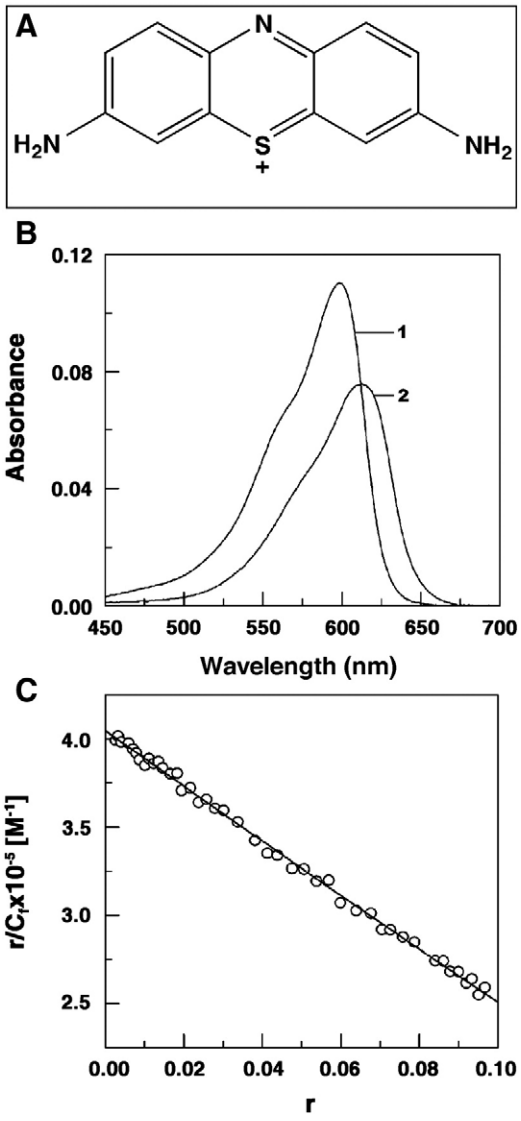

Fig. 1. (A) Chemical structure of thionine. (B) Absorption spectra of free (curve 1) and bound (curve 2) thionine (2 μMol) in presence of saturating concentrations of ML DNA. (C) representative Scatchard plot for the complexation obtained from spectrophotometric titration data. The solid line represent the best fit of the data points to the non-cooperative McGhee-von Hippel equation. All experiments were performed at 20 ± 0.5 °C in 50 mM sodium cacodylate buffer of pH 7.2. Values of K_i (binding constants) and n (number of excluded sites) are presented in Table 2.

the imino form is not. Although these data provide somewhat contradicting data on the GC specificity of thionine binding to linear double stranded DNA a recent study [17] by pressure tuning hole burning spectroscopy has conclusively shown an external stacking mode of binding of thionine to quadruplex structures. Thus, although some structural data is available, the same is not conclusive and the thermodynamics of thionine binding to DNA is not yet elucidated. In comparison to the closely related methylene blue [19–21] DNA binding aspects of thionine are scanty and inconclusive.

In this work we investigated the structural and thermodynamic aspects of thionine binding to three natural DNAs of varying base composition viz. from low GC (27%) to high GC (72%) content. We aimed to characterize the base specificity through spectroscopic studies and also elucidate the thermodynamics of the interaction to correlate the structural and energetic aspects to have a complete and unambiguous understanding of the interaction profile.

2. Materials and methods

2.1. Materials

Clostridium perfringenes (CP) DNA (Type XII, 27 mol% GC), herring testes (HT) DNA (Type XIV, 41 mol% GC) and *Micrococcus lysodeikticus* (ML) DNA (Type XI, 72 mol% GC) samples were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Each of these DNA exhibited characteristic ultraviolet absorption spectrum with an A_{260}/A_{280} ratio between 1.88 and 1.92 and an A_{260}/A_{230} ratio between 2.12 and 2.22. Concentration of these DNA duplexes was determined spectrophotometrically using molar extinction coefficient values of 12,600 $M^{-1}\,cm^{-1}$ for CP, 13,200 $M^{-1}\,cm^{-1}$ for HT and 13,800 $M^{-1}\,cm^{-1}$ for ML DNA expressed in terms of molarity of base pairs. DNA samples were sonicated to uniform size of about 280 ± 50 base pairs using a Labsonic 2000 sonicator (B. Braun, Germany) with a needle probe of 4 mm diameter as described previously [22]. After sonication, the DNA samples were dialyzed several times against the experimental buffer under sterile conditions. The nativeness of the DNA samples was confirmed from optical melting and differential scanning calorimetry where sharp melting profiles were observed. Thionine acetate (thionine/ligand in general) was a product of Sigma-Aldrich and was used as received. The sample was fairly soluble in aqueous buffers and hence its solution was freshly prepared each day in the experimental buffer and kept protected in the dark to prevent any light induced photochemical changes. The overall thionine concentration in each experiment was kept at the lowest possible to prevent dimer formation. The molar extinction coefficient (ε) and other optical properties of thionine are listed in Table 1. All other materials and chemicals were of analytical grade or better. All experiments were conducted at 20 °C in 50 mM sodium cacodylate buffer, pH 7.2. Deionized and doubled distilled water and analytical grade reagents were used throughout. pH measurements were made on a Cyberscan 2100 high precision bench pH meter (Eutech Instruments Pte. Ltd., Singapore). The buffer solution was filtered through Millipore membrane filters (Millipore, India Pvt. Ltd, Bangalore, India) of 0.45 μm pore size.

2.2. Absorption and fluorescence spectral studies and elucidation of binding parameters

A Jasco V 660 double beam double monochromator spectrophotometer (Jasco International Co, Tokyo, Japan) was used for absorbance studies at 20 ± 0.5 °C in 1 cm path length cuvettes using the methodologies described in details earlier [23,24]. Steady state fluorescence measurements were performed on a Shimadzu RF5301PC spectrofluorimeter (Shimadzu Corporation, Kyoto, Japan) in fluorescence free quartz cuvettes of 1 cm path length as described previously [23,25]. The excitation wavelength was 596 nm. The

Table 1
Summary of the optical properties of free and DNA bound thionine[a].

Parameter	ML DNA	HT DNA	CP DNA
Absorbance			
λ_{max} (free)	598	598	598
λ_{max} (bound)	612	611	610
λ_{iso}[b]	612	610	612
$\varepsilon_{f\ (at\ \lambda_{max})}$	54,200	54,200	54,200
$\varepsilon_{b\ (at\ \lambda_{max})}$	31,780 (598)	34,300 (598)	34,008 (598)
$\varepsilon_{iso\ (at\ \lambda_{iso})}$	37,460 (612)	42,050 (610)	38,440 (612)
Fluorescence			
λ_{max} (excitation)	596	596	596
λ_{max} (emission)	615	615	615
F_b/F_0	0.090	0.085	0.172

[a] Units: λ nm; ε (molar extinction coefficient) $M^{-1}\,cm^{-1}$. [b]Wavelengths at the isosbestic points. [c]F_0 and F_b are the fluorescence intensities of the free and completely bound thionine at 615 nm in each case.

emission maxima of thionine-DNA complexes were in the region 600–700 nm. All measurements were performed keeping an excitation and emission band pass of 3 nm at 20 ± 1.0 °C. The amount of free and bound thionine was determined as described in details previously [25].

Binding data obtained from spectrophotometric and spectrofluorimetric titrations were cast into Scatchard plots of r/C_f versus r. All the Scatchard plots revealed negative slope at low r values characteristic of non-cooperative binding isotherms and hence were analyzed using the following McGhee–von Hippel equation [26],

$$r / C_f = K_i(1-\mathrm{nr})[(1-\mathrm{nr})/\{1-(n-1)r\}]^{(n-1)} \quad (1)$$

where K_i is the intrinsic binding constant to an isolated binding site, and 'n' is the number of base pairs excluded by the binding of a single ligand molecule. All the binding data were analyzed using Origin 7.0 software (Microcal Inc., Northampton, MA, USA) to determine the best-fit parameters of K_i and 'n' as described in details earlier [24,27].

2.3. Determination of binding stoichiometry

Job plot [28,29] was constructed to determine the binding stoichiometry in each case from fluorescence spectroscopy results at 20 ± 1 °C as described previously [25,30]. At constant temperature, the fluorescence signal was recorded for mixture of solutions where the concentrations of both DNA and thionine were varied while the sum of their concentrations was kept constant. The difference in fluorescence intensity (ΔF) of thionine in the absence and presence of the DNAs was plotted as a function of the input mole fraction of the thionine. Break point in the resulting plot corresponds to the mole fraction of the bound thionine in the complex. The stoichiometry in terms of DNA–thionine $[(1-\chi_{\mathrm{thionine}})^{-}/^{-}\chi_{\mathrm{thionine}}]$ was obtained where χ_{thionine} denotes the mole fraction of thionine bound in each case. The results are average of at least three experiments.

2.4. Competition dialysis assay

Procedures developed by Chaires et al. [31,32] was followed generally for competition dialysis assay by dialyzing a 0.5 mL of each of the DNA solution (at identical concentration of 75 μM in monomeric unit) in separate 0.5 mL Spectra/Por® cellulose ester sterile DispoDialyzer units of 5 mm diameter (Spectrum Laboratories, Inc., CA, USA) against a 1 μM dialysate solution of thionine in a 200 mL beaker at 20 ± 2 °C. After a span of 24 h when equilibrium was achieved, the samples were carefully transferred to microfuge tubes. Dissociation of the thionine–DNA complex was done by adding appropriate volume of a 10% (w/v) of sodium dodecyl sulphate solution to make a final concentration of 1% (w/v) and the total concentration of the alkaloid (C_t) was determined by optical density measurements. The concentration of the free alkaloid (C_f) was determined by using an aliquot of the dialysate solution. The amount of bound alkaloid (C_b) was determined by the difference, ($C_b = C_t - C_f$). Data were plotted as a bar graph using Origin 7.0 software (MicroCal, Inc.; Northampton, MA, USA). The apparent binding constant (K_{app}) was calculated using the relation, $K_{app} = C_b/(C_f)(S_{total} - C_b)$ [32].

2.5. Fluorescence quenching studies

Quenching studies were carried out with the anionic quencher potassium ferrocyanide ($K_4[Fe(CN)_6]$). The quenching experiments were performed by mixing, in different ratios, two solutions, one containing KCl, the other containing $K_4[Fe(CN)_6]$, in addition to the normal buffer components, at a fixed total ionic strength. Experiments were performed at a constant P/D (DNA base pair/thionine molar ratio) monitoring fluorescence intensity as a function of changing concentration of the ferrocyanide as described previously [24]. The data were plotted as Stern–Volmer plots of relative fluorescence intensity (F_0/F) versus $[Fe(CN)_6]^{4-}$ concentration according to Stern–Volmer equation as described elsewhere [24,33].

2.6. Hydrodynamic studies

The viscosity of DNA–thionine complexes was determined by measuring the time of flow in a Cannon–Manning semi micro size 75 capillary viscometer (Cannon Instruments Company, State College, PA, USA) immersed in a thermostated bath maintained at 20 ± 1 °C as reported previously [22,23,33]. Flow times were measured in triplicate with an electronic stop watch to an accuracy of ±0.01 s. Relative viscosities for DNA either in the presence or absence of thionine were calculated from the relation,

$$\eta'_{sp}/\eta_{sp} = \left\{\left(t_{\mathrm{complex}} - t_0\right)/t_0\right\}/\{(t_{\mathrm{control}} - t_0)/t_0\} \quad (2)$$

where, η'_{sp} and η_{sp} are specific viscosities of the thionine-DNA complex and the DNA respectively; t_{complex}, t_{control}, and t_0 are the average flow times for the DNA–thionine complex, free DNA and buffer respectively. The relative increase in length, L/L_0, can be obtained from a corresponding increase in relative viscosity using the relation, $L/L_0 = (\eta/\eta_0)^{1/3} = 1 + \beta r$, where L and L_0 are the contour length of DNA in presence and absence of the thionine and η and η_0 are the corresponding values of intrinsic viscosity (approximated by the reduced viscosity $\eta = \eta_{sp}/C$ where C is the DNA concentration) and β is the slope when L/L_0 is plotted against r [25,34].

2.7. Spectropolarimetric studies

A JASCO J815 spectropolarimeter (Jasco International Co. Ltd., Tokyo, Japan) equipped with a Jasco temperature controller (model PFD 425 L/15) was used for all circular dichroic measurements at 20 ± 0.5 °C as reported earlier [22–24,33,34]. A rectangular strain free quartz cell of 1 cm path length was used. Instrument parameters for CD measurements were, scan rate of 50 nm/min. bandwidth of 1.0 nm, and sensitivity of 100 mdeg. The molar ellipticity values [θ] are expressed in terms of DNA base pair.

2.8. Optical thermal melting studies

Melting curves of DNA and DNA–thionine complexes were measured on a Shimadzu Pharmaspec 1700 unit equipped with a peltier controlled TMSPC-8 model accessory (Shimadzu Corporation, Kyoto, Japan) as described earlier [25,30]. DNA sample (20 μM) was mixed with varying concentrations of the thionine in the degassed buffer in to the eight chamber micro optical cuvette of 1 cm path length and the temperature of the microcell accessory was raised at a heating rate of 0.5 °C/min. The melting temperature (T_m) was taken as the midpoint of the melting transition as determined by the maxima of the first derivative plots.

2.9. Differential scanning calorimetry

The excess heat capacities as a function of temperature were measured on a Microcal VP-differential scanning calorimeter (DSC) (MicroCal, Inc., Northampton, MA, USA) as described previously [25,30]. In a series of DSC scans, both the sample and reference cells were loaded with buffer solution, equilibrated at 20 °C for 15 min and scanned from 40° to 120 °C at a scan rate of 60 °C/h. The buffer scans were repeated till reproducible and on cooling, the sample cell was rinsed and loaded with DNA solution and then with thionine–DNA complexes of different molar ratios and scanned. Each experiment was repeated twice with separate fillings. The DSC thermograms of excess heat capacity versus temperature were analyzed using the Origin 7.0 software to determine the calorimetric transition enthalpy

(ΔH_{cal}) as described earlier [25,30]. This calorimetrically determined enthalpy is model-independent and unrelated to the nature of the transition. The temperature at which excess heat capacity is maximum defines the transition temperature (T_m).

2.10. Isothermal titration calorimetry

A MicroCal VP-ITC unit (MicroCal, Inc., Northampton, MA, USA) using protocols developed in our laboratory and described in details previously [25,30,35,36] was used for all ITC experiments. A reverse titration protocol of injecting aliquots of DNA solution from a rotating syringe (290 rpm) into the calorimetric cell containing the thionine solution (1.4235 mL) under identical buffer conditions was adopted. Such a protocol enabled us to limit the concentration of thionine well below the aggregating conditions in calorimetry experiments. Corresponding control experiments to determine the heat of dilution of DNA were performed by injecting identical volumes of same concentration of the DNA into buffer. The area under each heat burst curve was determined by integration using the Origin 7.0 software to give the measure of the heat associated with the injection. The heat associated with each DNA-buffer mixing was subtracted from the corresponding heat of DNA–thionine reaction to give the heat of thionine–DNA binding. The heat of dilution of injecting the buffer into thionine solution alone was found to be negligible. The resulting corrected injection heats were plotted as a function of molar ratio and fit with a model for one set of binding sites and analyzed using Origin 7.0 software to provide the binding affinity (K), the binding (ΔH). The binding free energy (ΔG) and the entropic contribution to the binding ($T\Delta S$) were subsequently calculated from standard relationships described earlier [25,30].

3. Results and discussion

3.1. Solution properties of thionine

The solution properties of thionine were investigated initially to understand the concentrations under which the monomeric nature of the dye exists as this knowledge is a prerequisite for the study of interactions with DNA. Thionine obeys Lambert–Beer law till a concentration of 25 μM under the conditions of this experiment as revealed from absorbance and fluorescence studies. The spectroscopic properties of thionine remained unaltered in this concentration range under the influence of pH from 2–10, [Na^+] (Fig. S1, Supplementary data), in the range of 10–100 mM [Na^+] (Fig. S2, Supplementary data), and temperature from 10 to 50 °C (Fig. S3, Supplementary data). Thus under the conditions of the present experiment the solution properties of thionine remains unaltered and in full monomeric form.

3.2. Absorbance titration of thionine with DNAs and estimation of binding affinity

Thionine has characteristic visible absorption spectrum in the 450–700 nm region with a maximum at 598 nm that is convenient to monitor the interaction. Pronounced hypochromic and bathochromic effects were observed in this spectral region of thionine when mixed with increasing concentrations of the DNA duplexes, revealing strong intermolecular association. Such spectral changes have been usually interpreted to arise from a strong interaction between the π electron cloud of the interacting drug and the base pairs presumably due to intercalation. Representative absorption spectra of the free and fully bound thionine in presence of ML DNA are presented in Fig. 1A. The presence of a sharp isosbestic point enabled the assumption of a two state system consisting of bound and free thionine at any particular wavelength. A summary of the optical properties of thionine in the free and bound state with each DNA is presented in Table 1. Titration of a constant concentration of DNA with increasing concentration of thionine was performed with each of the DNA samples for evaluating the free and bound thionine at several inputs of the DNA as described in details earlier [24,25]. Binding data obtained from spectrophotometric titration was cast into the form of Scatchard plot of r/C_f versus r, where r is the number of moles of thionine bound per mole of DNA base pairs. In Fig. 1B, the Scatchard plot of thionine binding to ML DNA is depicted. The binding isotherm has no positive slope at low r values which indicate non-cooperativity enabling the fitting of the curves to a theoretical curve drawn according to the excluded site model of McGhee and von Hippel for a non-cooperative ligand binding system [26] to derive the best-fit parameters of the intrinsic binding constant to an isolated binding site and the number of base pairs excluded by the binding of a single thionine molecule. The binding affinity (K_i) of thionine to CP, HT and ML DNAs thus evaluated were 2.00×10^5 M^{-1}, 2.05×10^5 M^{-1} and 4.05×10^5 M^{-1} respectively. These values and the along with the numbers of excluded binding sites obtained in each case are depicted in Table 2. This result at the first instant suggests that the binding affinity of thionine to the GC rich ML DNA was higher than that with the AT rich CP DNA. Secondly the ratio of the binding constant to ML DNA and CP DNA gives a value of around 2.0-2.1, which indicates that only one GC base pair is preferred at the intercalation site. This is similar to many classical intercalators like sanguinarine, aristololactam-β-D-glucoside but different from others like daunorubicin that show more complex base specificity [6,37,38].

3.3. Fluorescence titration studies

Thionine has a strong intrinsic fluorescence with an emission spectrum in the 600–700 nm range with maximum at 615 nm when excited at 596 nm. Binding to the DNAs resulted in the quenching of the fluorescence that eventually leads to saturation of the binding sites in each case. A representative fluorescence pattern of the complexation of thionine with ML DNA is presented in Fig. 2. Large fluorescence change in each case is indicative of the strong association of molecules to these DNA structures resulting from an effective overlap of the bound molecules with the base pairs. This result also proposes the location of the bound molecules in a hydrophobic environment similar to an intercalated state. The binding isotherms revealed non-cooperative binding, a representative one for ML DNA complexation is presented in the inset of Fig. 2. The binding constants calculated from the fluorescence data as per the Scatchard analysis using the non-cooperative binding model of McGhee–von Hippel analysis (*vide supra*) yielded binding constants of 2.12×10^5, 2.62×10^5 and 4.35×10^5 M^{-1} for CP, HT and ML DNAs and these

Table 2
Binding parameters for thionine–DNA complexation from absorbance and fluorescence data[a].

DNA	GC mol%	Absorbance		Fluorescence		Competition dialysis
		$K_i\times10^{-5}(M^{-1})$	n	$K_i\times10^{-5}(M^{-1})$	n	$K_a\times10^{-5}(M^{-1})$
Clostridium perfringenes	27	2.00 ± 0.12	4.39	2.12 ± 0.15	3.95	1.12 ± 0.05
Herring testis	41	2.05 ± 0.08	3.15	2.62 ± 0.20	3.52	1.96 ± 0.08
Micrococcus lysodeikticus	72	4.05 ± 0.10	2.93	4.35 ± 0.12	3.39	5.14 ± 0.10

[a] Average of four determinations. Binding constants (K_i) and the number of occluded sites (n) refer to solution conditions of cacodylate buffer of 50 mM [Na^+], pH 7.2 at 20 °C. K_a is the apparent binding constant.

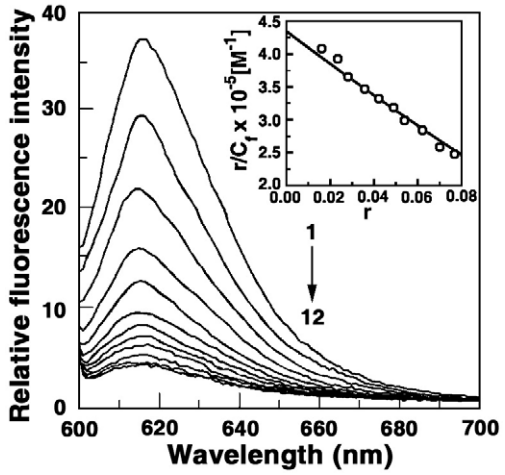

Fig. 2. Fluorescence spectral changes of thionine (1 μMol) with increasing concentrations of ML DNA (0–45 μM) at 20 ± 1 °C in 50 mM sodium cacodylate buffer of pH 7.2. Inset: representative Scatchard plot of the binding of thionine with ML DNA.

values are depicted in Table 2. It can be seen that these are in excellent agreement with the results obtained from spectrophotometric analysis.

3.4. Competition dialysis assay

Fig. 3 shows the result from the competition dialysis assay of the three DNA samples dialyzed against a common 1 μM thionine solution presented as a bar graph in which the concentration of thionine bound to each of the DNA sample is plotted. The highest binding in terms of more accumulation of the drug was found to be with ML DNA (9.5 μM) followed by HT DNA (3.7 μM) and the least with CP DNA (3.1 μM). The important conclusion that emerged from this experiment was the pronounced binding of thionine to ML DNA followed by HT representing the next most preferred sequence and CP DNA has the lowest binding preference. From the concentrations of bound dye, the apparent binding affinities (K_{app}) for the binding of thionine to the three DNAs was calculated and these values were, 1.12×10^5 1.96×10^5, 5.14×10^5 M^{-1} respectively for binding to CP, HT and ML DNAs. These results again indicate that the binding affinity of thionine was higher to the GC rich ML DNA and the affinity varied in the order ML>HT>CP DNA in complete agreement with that from the absorbance and fluorescence data.

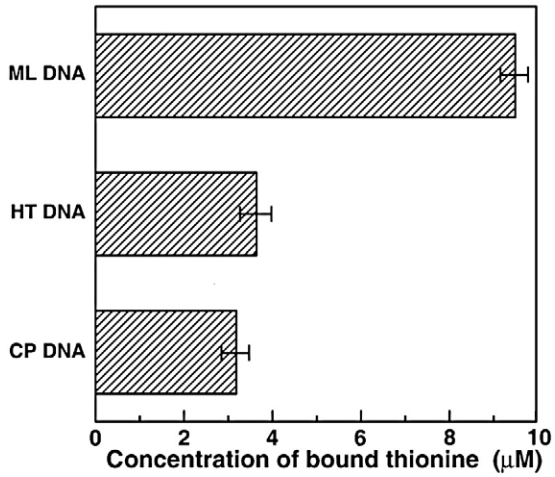

Fig. 3. The result of competition dialysis experiment in 50 mM sodium cacodylate buffer of pH 7.2 at 20 ± 2 °C. The concentration of thionine bound to each deoxyribonucleic acid sample is shown as a bar graph. The data given are average of four independent experiments under identical conditions.

3.5. Binding stoichiometry (Job plot)

The binding stoichiometry of thionine association to the DNAs and the possible number of binding sites were determined by continuous variation analysis (Job plot) in fluorescence. In Job plot, the ligand: DNA molar ratio is varied while the total molar concentration remains constant. The stoichiometry of binding is determined by the molar ratio where maximal binding is observed. The plot of difference in fluorescence intensity (ΔF) at 615 nm versus the mole fraction of thionine revealed a single binding mode in each case (figures not shown). From the inflection points, $\chi_{thionine} = 0.180$, 0.253 and 0.269 the number of base pairs of DNA bound per thionine can be estimated to be around 4.6, 3.0 and 2.7 respectively for CP, HT and ML DNAs. This is in good agreement with the number of occluded sites obtained from the non-cooperative McGhee–von Hippel analysis of the spectrophotometric and fluorimetric data (Table 2).

3.6. Fluorescence quenching and intrinsic viscosity studies for elucidation of the mode of binding

The mode of DNA binding of thionine was speculated previously to be intercalative [15–18] and we probed the same using fluorescence quenching experiments in presence of $[Fe(CN)_6]^{4-}$. Quenching experiments are straight forward and indicate the location of the bound molecules to be either on the outside or inside of the helix. An anionic quencher would not be able to penetrate the negatively charged barrier around the helix and if the bound thionine molecules are buried within the DNA helix by intercalation little or no change in its fluorescence is expected. Stern–Volmer plots for the quenching of thionine–DNA complexes are shown in Fig. 4A. Results clearly indicate that free thionine molecules are quenched efficiently. Very little quenching was observed in case of complexes with ML and HT DNA and more quenching for molecules bound to CP DNA indicating the bound thionine molecules in the former cases are located in a relatively more protected environment compared to CP DNA, presumably more deeply and or strongly intercalated. The Stern–Volmer quenching constants calculated for thionine and its complexes with CP, HT and ML DNAs were 41, 39, 28, and 23 L/mol respectively. From these results it can be inferred that the bound thionine molecules are sequestered away from the solvent indicating strong intercalative binding to ML and HT while that in CP DNA is accessible to the quencher to some extent.

To further substantiate the intercalative binding mode of the thionine to the DNAs, the viscosity of the DNA solution was measured in presence of increasing concentration of the thionine. Hydrodynamic measurements are very sensitive to length changes and stiffening in rod like DNA and may be regarded as diagnostic test for elucidating the binding mode. Lerman's original hypothesis predicted that the viscosity of a rod like nucleic acid solution increases upon complexation with intercalators [39]. This is due to the fact that the axial length of the nucleic acid enhances and it becomes more rigid. The viscosity increase occurs as both the factors enhance the frictional coefficient. Viscosity results are expressed as length enhancement (Fig. 4B) per base pair estimated with respect to a standard value (β) of 1 corresponding to a length enhancement of 0.34 nm. The β values for thionine binding to CP, HT and ML DNAs were 0.69, 0.77 and 0.86 nms respectively. Thus, a stronger intercalation scenario may be envisaged for the binding to the base pair sequences of ML and HT DNAs while a weaker intercalation process may be assigned for the binding to the CP DNA in agreement with the fluorescence quenching results.

3.7. Optical thermal melting and differential scanning calorimetric studies

The ability of small molecules to enhance the stability of double stranded DNA on binding is studied through optical melting and by

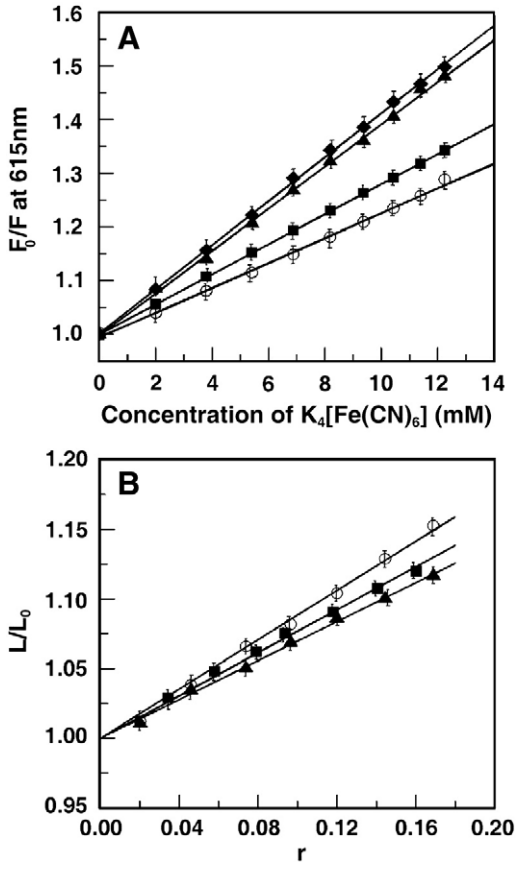

Fig. 4. (Upper panel) Stern–Volmer plots for the quenching of thionine (♦) and complexes of thionine–CP DNA (▲), thionine–HT DNA(■), and thionine–ML DNA (○) with increasing concentrations of $[Fe(CN)_6]^{-4}$ ion in 50 mM sodium cacodylate buffer, pH 7.2, at 20 ± 1 °C. Concentration of thionine, DNAs and K^+ ion were kept constant. (Lower panel) A plot of increase in helix contour length (L/L_0) versus r for the complexation of CP DNA(▲), HT DNA(■) and ML DNA(○) with thionine in 50 mM sodium cacodylate buffer, and pH 7.2, at 20 ± 0.5 °C.

differential scanning calorimetry. Native CP, HT and ML DNAs melted at 72.0°, 79.0° and 92.0 °C respectively under the salt conditions employed in this study. The binding of thionine stabilized all the three DNAs and ΔT_m values of about 6.3°, 8.5° and 9.4 °C respectively were obtained under saturating conditions with CP, HT and ML DNAs. The optical melting profiles are presented in Fig. 5A, B, C. This result further reiterates the strong binding of thionine to these DNA polymers. DSC studies also revealed similar stabilization temperatures (Fig. 5D, E, F). The binding constants of thionine–DNA association was calculated from the melting data using the equation derived by Crothers [40],

$$1/T_m^0 - 1/T_m = (R/n\Delta H_{wc}) \ln(1 + K_{Tm}\alpha) \quad (3)$$

where T^0_m is the optical melting temperature of the DNAs in the absence of the drug, T_m is the melting temperature in presence of saturating amounts of the drug, ΔH_{wc} is the enthalpy of DNA melting, R is the universal gas constant (1.987 cal K^{-1} mol^{-1}), K_{Tm} is the drug binding constant at the T_m, α is the free drug activity, that may be estimated by one half of the total drug concentration, and n is the site size of the drug binding. The calculated apparent binding constant at the melting temperature may be extrapolated to a reference temperature, say 20 °C, using the standard relationship, $\delta[\ln(K_{obs})]/\delta(1/T) = -(\Delta H_b/R)$ where K_{obs} is the drug binding constant at the reference temperature T (in Kelvin) and ΔH_b is the binding enthalpy which may be directly determined from an isothermal titration calorimetry experiment (*vide infra*). The binding constants (K_{obs}) calculated from these data were found to be 1.65×10^5, 3.21×10^5 and 5.60×10^5 M^{-1} respectively for CP, HT and ML DNAs at 20 °C (Table 3). These values are of the same order and close to the values of K_i obtained from spectroscopic studies.

3.8. Spectroscopic study using circular dichroism

The intrinsic CD spectra of the DNA duplexes displayed a canonical B-form conformation characterized by a large positive band in the 270–280 nm and a negative band at 245 nm although there are small differences in the ellipticity and wavelength maxima. Conformational changes associated with the binding of thionine with these DNAs were investigated from circular dichroic studies. The CD bands of DNA are caused due to the stacking interactions between the bases and the helical structure that provide asymmetric environment for the bases. Thionine on the other hand is achiral and does not have any intrinsic optical activity but may acquire optical activity (induced CD) on binding to the helical organization of DNA. To record thionine-induced changes in the DNA conformation, the CD spectra in the 210–400 nm regions were recorded in presence of varying D/P (thionine/DNA base pair molar ratio) values. In presence of thionine, the ellipticity of the long wavelength positive band of the DNAs increased as the interaction progressed with a slight red shift in the wavelength maximum till saturation was achieved at a D/P of 1.0. The CD spectral data are presented in Fig. 6A–C. To examine the conformational aspects in more detail, induced CD spectra in the 300–700 nm region were recorded where neither the DNAs nor thionine has any CD spectra. This region exclusively presents the CD induced in the bound thionine molecules essentially on binding asymmetrically to the chiral DNA helix. Thionine molecules strongly bound may acquire induced circular dichroic characteristics in the asymmetric DNA environment, which was already visible in each case from intrinsic CD studies (Fig. 6A–C). A negative induced CD band appears near 310 nm. Further, strong induced CD bands appear on either side of the wavelength maximum of absorption of thionine (600 nm) with each of the DNA with the positive wavelength maximum around 630 nm and the negative maximum around575 nm. This may be attributed to an exciton splitting mechanism arising due to the effective interaction of the transition moments of intercalated thionine with that of the base pairs whereby the isohelical arrangement of the drug molecules on the DNA results in an asymmetric arrangement of the dye chromophores. Similar CD signals indicate similar orientation of thionine on these DNAs but the differences in the intensities indicate that the binding is sensitive to the base composition, being higher with the GC rich ML DNA, followed by HT DNA and lowest with the AT rich CP DNA. The ellipticity of these bands initially increases and then decreased with decreasing P/D in each case. Thus the magnitude of the induced CD spectral bands may suggest differences in the strength of the bound molecule inside the helical organization of the DNA duplexes. It is pertinent to observe that the base pairing schemes and the dipole moments of the base pairs of these DNAs are different and their interaction with thionine is expected to be different leading to the different manifestation of the induced CD band ellipticities although the orientations may be similar. This region of the induced CD generated in thionine on interaction with DNA has been previously characterized [41] but the interpretations have been speculative. In analogy with such interpretations, the band in the long wavelength region (>400 nm) with maximum around 630 nm with these double helical DNAs may be inferred to arise due to the effective interaction of the transition moments of intercalated thionine with that of the base pairs. Based on this, the intercalation of thionine to ML DNA appears to be stronger than with the HT and CP DNA and is in confirmation with the results from other spectroscopic experiments.

3.9. Calorimetric determination of the thermodynamic parameters

Top panel of Fig. 7 presents the representative primary data from the isothermal calorimetric titration of each of the DNA samples (500 μM)

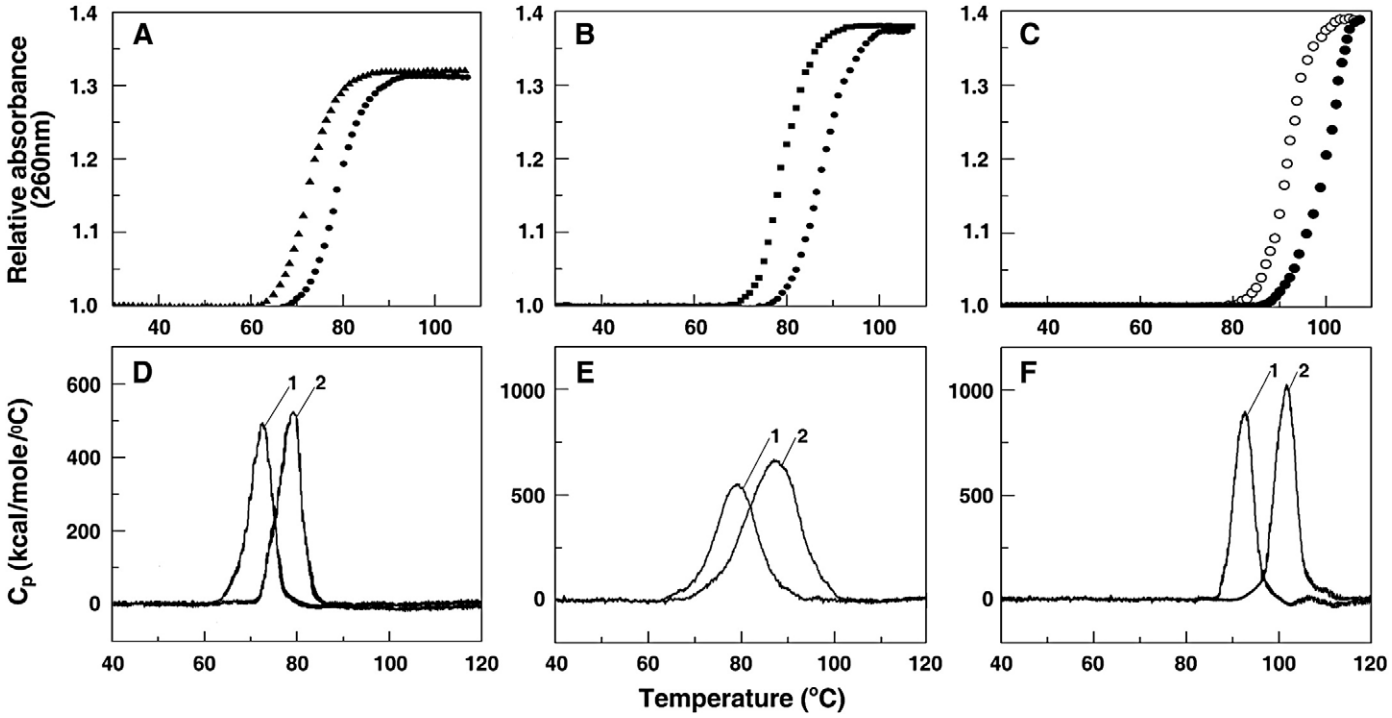

Fig. 5. Optical thermal melting profiles of (A) CP DNA(▲), (B) HT DNA(■), (C) ML DNA (○) and CP DNA-thionine complex, HT DNA–thionine complex, ML DNA–thionine complex (●) (upper panels). DSC profiles, of (D) CP DNA, (E) HT DNA, (F) ML DNA (curve 1) and CP DNA–thionine complex, HT DNA–thionine complex, and ML DNA–thionine complex (curve 2) (lower panels).

into a solution of thionine (10 μM) at 20 °C. Each heat burst curve in the figures corresponds to a single injection. These injection heats were then corrected by subtracting the respective dilution heats derived from the injection of identical amounts of DNA into the buffer alone (shown in the upper panel, curves off set for clarity). The bottom panels in Fig. 7 presents the resulting corrected heats plotted against the molar ratio. The data points here are the actual experimental points and the continuous line is the calculated best fit to the data. The binding in each case was characterized by exothermic heats. Larger heat is released in ML DNA compared to CP DNA. The ITC data were fit to a single site model as the integrated heat data showed single binding event in each case. The results of the ITC experiments are presented in Table 5. The binding affinity values obtained from ITC are in the order of 10^5 M^{-1}, which follow the same trend as that obtained from spectroscopic studies, being highest for ML DNA and the values varying in the order ML>HT>>CP DNA. With the HT and CP DNA the binding was favored by enthalpy and entropy contributions, the entropy contribution being large in CP DNA. The large negative enthalpy of the binding and positive entropy contributions observed here particularly are generally typical for intercalative interaction of small molecules to nucleic acids [21,42]. The strong positive entropy term in each case is suggestive of the disruption and release of water molecules on intercalation of thionine into the DNA helix and is more in the AT rich CP DNA. It is known that the AT base pairs are associated with more water of hydration than the GC pairs. The binding affinity values observed form ITC may be equated with the K_i values from spectroscopic binding experiments, which are closely comparable in each case. These values are also comparable to that evaluated from thermal melting data.

3.10. Dependence of binding on the ionic strength on the medium

To provide insight into the nature of the binding, the effect of salt concentration on the binding in ITC in conjunction with van't Hoff analysis was performed in the case of HT DNA as a representative case. The following relation between the binding constant and $[Na^+]$ ion concentration has been described previously [43] viz. $\delta\log(K)/\delta\log([Na^+]) = -Z\varphi$, where Z is the apparent charge on the bound ligand and φ is the fraction of $[Na^+]$ bound per DNA phosphate group. The slope of the linear plot of ln K versus ln $[Na^+]$ (not shown) gave a value of −0.64 for HT DNA. A low value of the slope compared to the theoretical value of 1.0

Table 3
Thermal melting data and binding constants from melting data at saturating concentrations of thionine with DNA[a].

DNA system	T_m (°C) (Optical melting)	T_m (°C) (DSC)	ΔH_{cal} (kcal /mol)	ΔH_v (kcal /mol)	K_{Tm} $\times 10^{-5}$ (M^{-1})	K_{obs} $\times 10^{-5}$ (M^{-1})
ML DNA	92.01	92.44	4.84×10^3	2.01×10^5		
ML DNA + thionine ($D/P=0.5$)	101.41	101.5	5.81×10^3	1.97×10^5	1.16	5.59
ΔT_m	9.40	9.06	–	–		
HT DNA	78.92	78.96	6.41×10^3	8.47×10^4		
HT DNA + thionine ($D/P=0.5$)	87.41	86.77	9.72×10^3	7.11×10^4	1.22	3.21
ΔT_m	8.49	7.81	–	–		
CP DNA	72.16	72.28	3.06×10^3	1.51×10^5		
CP DNA + thionine ($D/P=0.4$)	78.48	78.43	3.24×10^3	1.47×10^5	0.84	1.65
ΔT_m	6.32	6.15	–	–		

[a] Melting stabilization of DNAs in presence of saturating amounts of thionine in cacodylate buffer of 50 mM $[Na^+]$, pH 7.2. The data are average of four determinations. K_{Tm} is the binding constant at the melting temperature and K_{obs} is the drug binding constant at 20 °C determined using equations described in the text. ΔH_{cal} and ΔH_v were obtained from the DSC data.

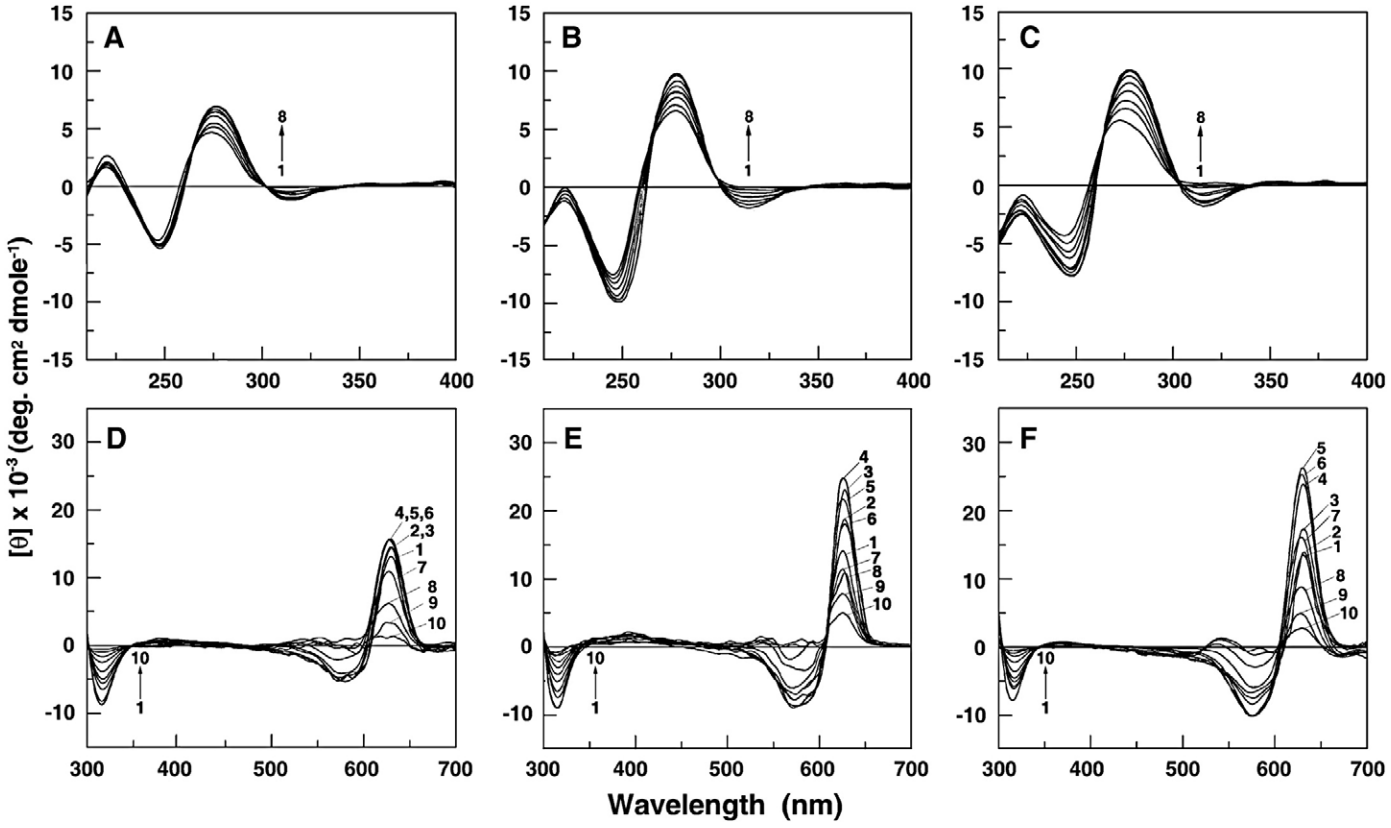

Fig. 6. Representative intrinsic circular dichroic spectra (upper panels) of 32.5 μM of (A) CP DNA, (B) HT DNA and (C) ML DNA treated with 0.0, 3.25, 9.75, 16.25, 19.5, 26.0, 29.75, and 32.0 μM of thionine (curves 1–8). The expressed molar ellipticity (θ) is based on the DNA concentrations. Representative induced circular dichroic spectra (lower panels) of 25 μM of thionine treated with 3.12, 6.25, 12.5, 25, 50, 75, 100, 150, 200 and 250 μM of (D) CP DNA (E) HT DNA and (F) ML DNA (curves 1–10). The expressed molar ellipticity is based on the thionine concentration.

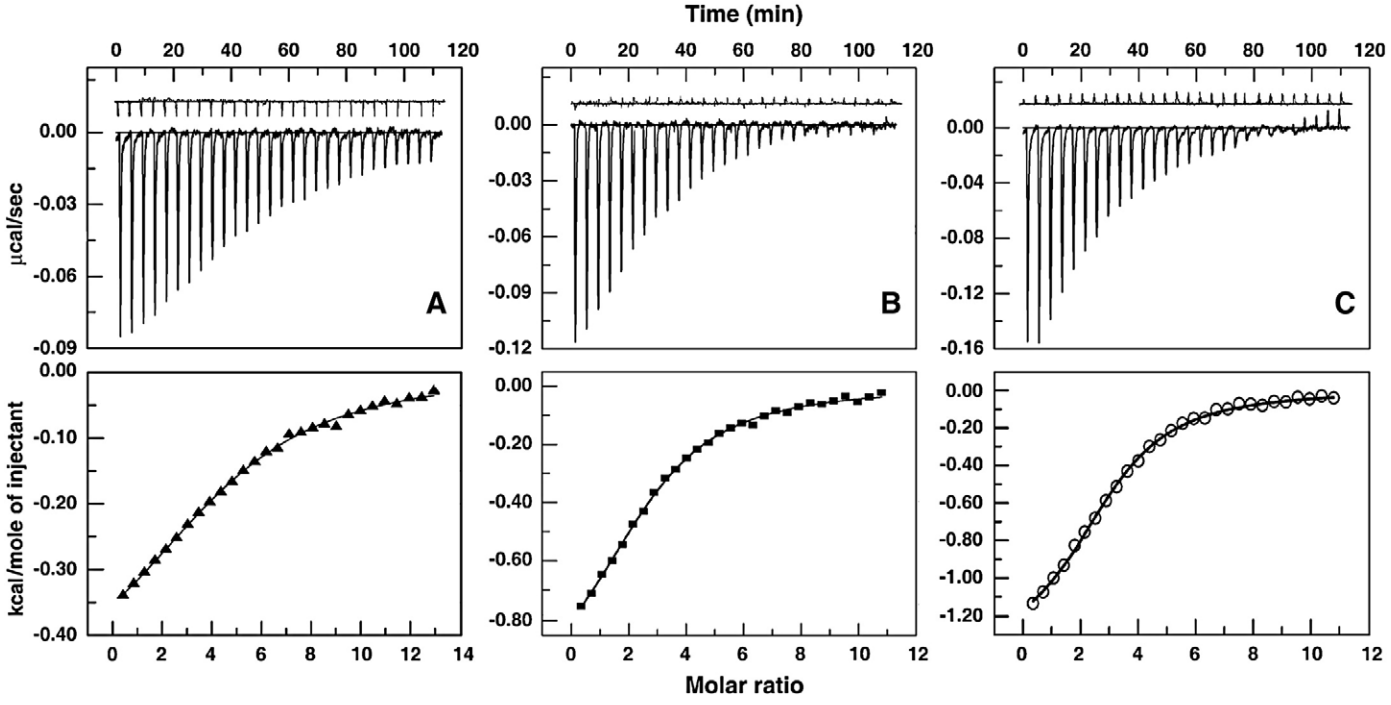

Fig. 7. Representative ITC profiles for the titration of (A) CP DNA, (B) HT DNA and (C) ML DNA with thionine at 20 °C in 50 mM sodium cacodylate buffer of pH 7.2. The top panels represent the raw data for the sequential injection of DNA into thionine and the bottom panels show the integrated heat data after correction of heat of dilution against the molar ratio of DNA/drug. The data points (▲, thionine–CP DNA; ■, thionine–HT DNA; ○, and thionine–ML DNA) reflect the experimental injection heat, which were fitted to one site model, and the solid lines represent the best-fit data. The binding constants (K) and other thermodynamic parameters are depicted in Table 5.

Table 4
Thermodynamic parameters for the association of thionine with the HT DNA at four different salt concentrations[a].

$[Na^+]$ (mM)	$K\times10^{-5}$ (M^{-1})	N	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)	$-\Delta G^{pe}$ (kcal/mol)	$-\Delta G^{t}$ (kcal/mol)
10	6.25	0.45	−3.15	4.67	−7.82	1.72	6.10
20	4.42	0.39	−3.08	4.54	−7.62	1.47	6.15
50	2.32	0.37	−3.04	4.19	−7.23	1.11	6.12
100	1.49	0.32	−2.76	4.22	−6.98	0.86	6.12

[a] All the data in this table are derived from ITC experiments conducted in cacodylate buffer of different $[Na^+]$, pH 7.2 and are average of four determinations. K and ΔH values were determined from ITC profiles fitting to Origin 7 software as described in the text. The values of ΔG and $T\Delta S$ were determined using the equations $\Delta G = -RT\ln K$, and $T\Delta S = \Delta H - \Delta G$. All the ITC profiles were fit to a model of single binding sites.

for monocationic intercalators suggests weaker electrostatic contacts of thionine with DNA. The observed free energies of the interaction are in the range 7.0 to 8.0 kcal/mol (Table 4). From the dependence of K on $[Na^+]$, the observed free energy can be partitioned between the polyelectrolytic (ΔG^{pe}) and non polyelecrolytic (ΔG^{t}) contributions. The contribution to the free energy from the electrostatic interaction (polyelectrolytic) can be quantitatively determined from the relationship, $\Delta G^{pe} = Z\varphi RT\ln([Na^+])$, where $Z\varphi$ is the slope of the van't Hoff plot. The contribution of ΔG^{pe} has been determined to be around 1.91, 1.63, 1.24 and 0.96 kcal/mol at 10, 20, 50 and 100 mM $[Na^+]$ (Table 4) which are only about 24.45, 21.47, 17.29 and 13.49% of the total free energy change at each salt concentration. Thus, at 10 mM $[Na^+]$ where electrostatic interaction may be predominant, one fourth of the contribution to the free energy is from the electrostatic forces while at 100 mM$[Na^+]$ where the ionic interactions are presumed to be lower and the contribution to the free energy is about one eighth. It is pertinent to note that a significant contribution of the ionic forces to the binding of thionine to viral DNA/RNA was also suggested previously [13].

3.11. Heat capacity changes

The heat capacity changes (ΔCp) of small molecule–DNA binding interactions can be determined from the temperature dependence of the binding enthalpy using the standard relationship, $\Delta Cp = \delta(\Delta H)/\delta T$. This information provides valuable insights into the type and magnitude of forces involved in the binding process. Temperature dependent ITC was performed at four temperatures in the range of 10–30 °C and the thermodynamic parameters were elucidated at these temperatures. The association constant varied from 4.03×10^5 to 1.53×10^5 M^{-1} for CP, from 5.61×10^5 to 1.61×10^5 M^{-1} for HT and from 6.15×10^5 M^{-1} to 3.05×10^5 M^{-1} for ML DNA. The temperature dependence of the enthalpy change was used to estimate change in heat capacity (ΔCp). The variation of ΔH with temperature for the three DNAs are presented in Fig. 8. The slope of the lines revealed values of −101, −133 and −208 cal/mol K respectively for the binding to CP, HT and ML DNA (Table 5). A large ΔCp value is usually associated with changes in hydrophobic or polar group hydration and considered as indicator of a dominant hydrophobic effect in the binding process. Negative heat capacity values have been observed for a variety of small molecules binding to DNA and RNA [21,23,27,35,42]. Change in solvent accessible surface area has been shown to be large component of ΔCp [44–46]. Further, structured water like the water of hydrophobic hydration can be associated with large heat capacity and the release of such water associated with the transfer of the non-polar groups into the interior of the helix can contribute a negative term to the ΔCp. First of all, the values of ΔCp are non-zero indicating temperature dependence of the enthalpy change. Secondly, the values of ΔCp in almost all cases fall within the range of 100–500 cal/mol K or are very close to the lower limit that is frequently observed for both ligand–nucleic acid and ligand–protein interaction [47,48]. The higher values of ΔCp for thionine binding to ML DNA compared to others most likely suggest substantial conformational change in this DNA structure that was reflected in circular dichroic study as well. The significant differences in the ΔCp values may also indicate differences in the release of structured water consequent to the transfer of non-polar groups into the inside of the DNA helix. Four types or modes of DNA recognition by small molecules viz. sequence specific, nonspecific, minimal sequence specific and structure specific were discussed by Murphy and Churchill [49]. Small negative ΔCp values are considered to be generally associated with a minimal sequence specific binding. Therefore, the slightly negative and non-zero ΔCp values that were observed for thionine–DNA complexation appear to denote structure specific binding. Generally, for DNA intercalators and groove binding molecules a large hydrophobic contribution to the binding free energy is expected due to their aromatic ring system and binding should be energetically favourable [48]. The free energy contribution from the hydrophobic transfer step of binding of these molecules may be calculated from the relationship [50], $\Delta G_{hyd} = 80(\pm10)\times\Delta Cp$. The ΔG_{hyd} values for thionine binding to CP, HT and ML DNAs were deduced to be −8.1, −10.7 and −16.7 kcal/mol, that are well within the range that was observed for typical intercalating DNA and RNA intercalating molecules [21,24,36,42,44,48,51].

3.12. Enthalpy–entropy compensation

Another significant feature observed in the thermodynamics of thionine–DNA interaction is the enthalpy–entropy compensation phenomenon that was observed in several drug–DNA interaction studies. Fig. 9 depicts the variations of ΔG and ΔH as a function of $T\Delta S$ for thionine binding to the three DNAs investigated. It can be observed that the variation of enthalpy change with entropy contribution was linear in each case. The values of slope that is $\delta\Delta H/\delta(T\Delta S)$ were 0.96, 0.97 and 1.03 respectively for CP, HT and ML DNAs. Since the value is very close to unity, it shows complete compensation in each case. Enthalpy–entropy compensation is often linked to the solvent reorganization accompanying binding interactions [52,53]. Linear relationship of enthalpy change with $T\Delta S$ with slope near unity is an indication of complete compensation and this occurs in systems with ΔCp not equal to zero and $\Delta Cp > \Delta S$. The data obtained here suggests almost complete compensation in all the cases.

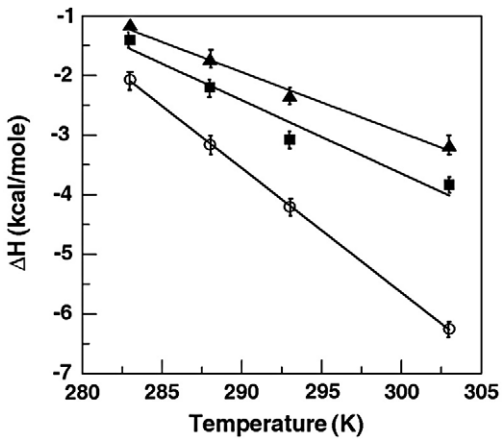

Fig. 8. Plot of variation of enthalpy of binding (ΔH) with temperature for the binding of thionine with (A) CP DNA (▲) (B) HT DNA (■) and (C) ML DNA (○) 50 mM sodium cacodylate buffer of pH 7.2.

Table 5
Temperature dependent isothermal titration calorimetric data for the binding of thionine to CP, HT and ML DNA[a].

DNAs	T (K)	$K\times10^{-5}$ (M^{-1})	ΔG (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔC_p (cal/mol K)	ΔG_{hyd} (kcal/mol)
Clostridium perfringenes	283	4.03 ± 0.06	−7.26	−1.17	6.08	−101.1	−8.1
	288	3.01 ± 0.07	−7.22	−1.75	5.47		
	293	2.14 ± 0.04	−7.14	−2.36	4.78		
	303	1.53 ± 0.07	−7.20	−3.20	3.99		
Herring testis	283	5.61 ± 0.06	−7.29	−1.26	6.03	−133.2	−10.7
	288	4.62 ± 0.03	−7.36	−2.10	5.27		
	293	2.32 ± 0.12	−7.27	−3.11	4.16		
	303	1.61 ± 0.07	−7.25	−3.92	3.33		
Micrococcus lysodeikticus	283	6.15 ± 0.02	−7.51	−2.07	5.43	−208.4	−16.7
	288	5.21 ± 0.01	−7.54	−3.16	4.38		
	293	4.26 ± 0.01	−7.54	−4.20	3.34		
	303	3.05 ± 0.01	−7.61	−6.25	1.36		

[a] All the data in this table are derived from ITC experiments conducted in 50 mM sodium cacodylate buffer, pH 7.2 and are average of four determinations. K and ΔH values were determined from ITC profiles fitting to Origin 7 software as described in the text. The values of ΔG and $T\Delta S$ were determined using the equations $\Delta G = -RT\ln K$, and $T\Delta S = \Delta H - \Delta G$. All the ITC were fit to a model of single binding sites.

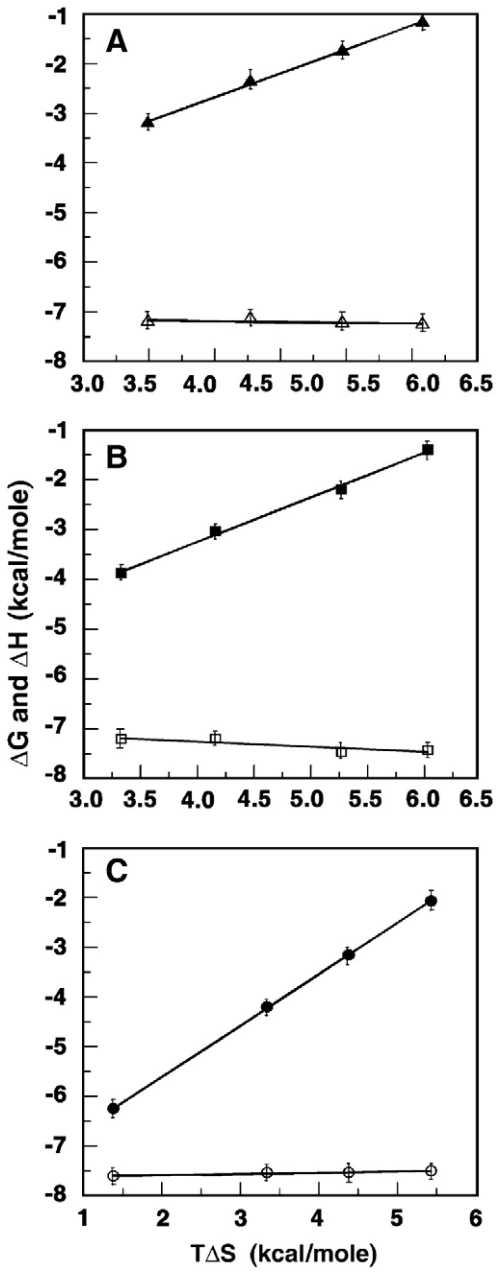

Fig. 9. Plot of ΔG (open symbols) and ΔH (closed symbols) versus $T\Delta S$ for the binding of thionine with (A) CP DNA (Δ, ▲) (B) HT DNA (□, ■) and (C) ML DNA (○, ●).

4. Conclusions

This study presents complete structural and thermodynamic results of the intercalation reaction of the phenothiazinium dye thionine with deoxyribonucleic acids of varying base compositions using various biophysical and calorimetric tools. The results of the structural studies reveal that thionine binds strongly with the DNAs; the strength of the binding being stronger with the GC rich *M. lysodeikticus* DNA and weaker with the AT rich *C. perfringenes* DNA. The binding to the DNAs was non-cooperative that resulted in significant perturbation of the conformation of the DNAs as well as thermal stabilization. The binding also resulted in induction of optical activity in the bound dye molecules. Fluorescent quenching and hydrodynamic studies revealed that the binding of thionine to the DNAs was intercalative, again stronger with the GC rich *M. lysodeikticus* DNA compared to the AT rich *C. perfringenes* DNA. Thermodynamics of the interaction revealed that the binding was favored by both negative enthalpy and positive entropy changes, but to different extents and showed enthalpy–entropy compensation behaviour. Negative heat capacity changes in all the systems are correlated to the involvement of significant hydrophobic forces in the complexation. The binding of thionine to the AT rich *C. perfringenes* DNA was favored by higher entropic contribution compared to that with the GC rich *M. lysodeikticus* DNA indicating the stronger perturbation of the water structure associated with the AT sequences. Taken together, the results unequivocally provide evidences for the intercalative GC specific DNA binding of thionine and advance our knowledge on the energetics of interaction of small molecules to DNA that may useful for designing more potential DNA binding therapeutic molecules.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2010.02.015.

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